

U.S. Application No.
Pending

International Application No.
PCT/BE99/00105

09/762992
Rec'd PCT/PTO 14 FEB 2001
Attorney Docket No.
VANM201.001APC
-COPY-

Date: February 14, 2001

Page 1

**TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 USC 371**

International Application No.: PCT/BE99/00105
International Filing Date: August 14, 1999
Priority Date Claimed: August 14, 1998
Title of Invention: NUCLEOTIDE AND/OR AMINO-ACID SEQUENCE CONTROLLING THE
EXPRESSION OF A XYLANASE PROMOTER-OPERATOR NUCLEOTIDE
SEQUENCE
Applicant(s) for DO/EO/US: Fabrizio Giannotta, Sebastien Rigali, Jean Dusart

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. (X) This is a **FIRST** submission of items concerning a filing under 35 USC 371.
2. (X) This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 371(b) and PCT Articles 22 and 39(1).
3. (X) A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
4. (X) A copy of the International Application as filed (35 USC 371(c)(2))
 - a) () is transmitted herewith (required only if not transmitted by the International Bureau).
 - b) (X) has been transmitted by the International Bureau.
 - c) () is not required, as the application was filed in the United States Receiving Office (RO/US).
5. (X) Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3))
 - a) () are transmitted herewith (required only if not transmitted by the International Bureau).
 - b) () have been transmitted by the International Bureau.
 - c) () have not been made; however, the time limit for making such amendments has NOT expired.
 - d) (X) have not been made and will not be made.
6. (X) An oath or declaration of the inventor(s) (35 USC 371(c)(4)).
7. (X) A copy of the International Preliminary Examination Report with any annexes thereto, such as any amendments made under PCT Article 34.
8. (X) An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
9. (X) A FIRST preliminary amendment.

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10. (X) International Application as published:
a. (X) Publication Cover Sheet
b. (X) 15 pages of disclosure
c. (X) Sequence Listing in 5 pages
d. (X) 3 pages of drawings
e. (X) International Search Report
11. (X) Small Entity status is established.
12. (X) PCT Form PCT/IPEA/402.
13. (X) PCT Form PCT/IB/308.
14. (X) PCT request form.
15. (X) A return prepaid postcard.
16. (X) The following fees are submitted:

				FEES
BASIC FEE				\$860
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total Claims	26 - 20 =	6 ×	\$18	\$108
Independent Claims	1 - 3 =	0 ×	\$80	\$0
TOTAL OF ABOVE CALCULATIONS				\$968
Reduction by 1/2 for filing by small entity (if applicable). Verified Small Entity statement must also be filed. (NOTE 37 CFR 1.9, 1.27, 1.28)				\$484
TOTAL NATIONAL FEE				\$484
TOTAL FEES ENCLOSED				\$484

17. (X) A check in the amount of \$484 to cover the above fees is enclosed.
18. (X) Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40 per property.
19. (X) The Commissioner is hereby authorized to charge only those additional fees which may be required, now or in the future, to avoid abandonment of the application, or credit any overpayment to Deposit Account No. 11-1410. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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SEND ALL CORRESPONDENCE TO:

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021401

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Printed Name _____

34,115

Registration Number

09/762992

02 Rec'd PCT/PTO 14 FEB 2001

VANM201.001APC

PATENT

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5/1/01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Giannotta, et al)	Group Art Unit unknown
Int'l Appl. No.	:	PCT/BE99/00105)	
Int'l Filing)	
Date	:	August 14, 1999)	
For	:	NUCLEOTIDE AND/OR)	
		AMINO-ACID SEQUENCE)	
		CONTROLLING THE)	
		EXPRESSION OF A)	
		XYLANASE PROMOTER-)	
		OPERATOR NUCLEOTIDE)	
		SEQUENCE)	
Examiner	:	Unknown)	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Preliminary to Examination on the merits, please amend the above-captioned patent application as follows:

IN THE SPECIFICATION

On page 1 of the Specification, line 12, after the Title of the Invention, please insert ~~This~~
is the U.S. National Phase of International Application PCT/BE99/00105, filed August 12, 1999,
which claims priority of U.S. Provisional Application, 60/096,556, filed August 14, 1998.--

On page 3, line 31, please cancel the word "pairs" and substitute in its place --pair--.

On page 5, line 10, please cancel the word "hybridisation" and substitute in its place --
hybridization--.

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On page 5, line 15, please cancel the word "hybridisation" and substitute in its place -- hybridization--.

On page 5, line 16, please cancel the word "hybridisation" and substitute in its place -- hybridization--.

On page 5, line 20, please cancel the word "hybridised" and substitute in its place -- hybridized--.

On page 5, line 22, please cancel the word "hybridisation" and substitute in its place -- hybridization--.

On page 6, line 9, after the word "described.", please insert--[SEQ ID NO:4]--.

On page 9, line 2, in Table 1, after the sequence "CGAAACTGTTGA", please insert --[SEQ ID NO:7]--.

On page 9, line 3, in Table 1, after the sequence "TTTCCGAAAGTTTGCC", please insert --[SEQ ID NO:8]--.

On page 9, line 4, in Table 1, after the sequence "TCGAAACTTTTCG", please insert --[SEQ ID NO:9]--.

On page 9, line 5, in Table 1, after the sequence "t CGAAA g c c", please insert --[SEQ ID NO:10]--.

On page 9, line 8, after the word, "sequence", please insert -- TXXCGAAAXXGXCXC[SEQ ID NO:10]--.

On page 11, line 15, please cancel the word "biotine" and substitute in its place --biotin--.

On page 11, line 16, please cancel the word "hybridisation" and substitute in its place -- hybridization--.

On page 12, line 25, after the word "fragment", please cancel the word "SEQ ID NO 5:" and substitute in its place --SEQ ID NO:6--.

On page 12, line 28, after the sequence, please cancel "(or SEQ ID NO 4)" and substitute in its place --(or SEQ ID NO:5)--.

On page 12, line 32, please cancel the words "streptomycine/spectinomycine" and substitute in its place --streptomycin/spectinomycin--.

On page 13, line 19, please cancel the word "foreigner" and substitute in its place -- foreign--.

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On page 14, line 1, please cancel the word "CLAIMS" and substitute in its place -WHAT IS CLAIMED IS:--.

IN THE CLAIMS

Please amend the claims as follows:

1. (Amended) [Isolated and purified genetic sequence (1) controlling in trans]An isolated polynucleotide which controls the expression of a xylanase promoter-operator nucleotide sequence in trans, comprising [(2)] at least about 100 nucleotides of SEQ ID NO:1, its complement, or a homolog, wherein said homolog controls the expression of said xylanase promoter-operator.
2. (Amended) [Isolated and purified genetic sequence]The isolated polynucleotide according to claim 1, [being a nucleotide sequence which presents]with more than 60% homology with the nucleotide sequence SEQ ID NO:1 or its complementary strand.
3. (Amended) [Isolated and purified genetic sequence]The isolated polynucleotide genetic sequence according to claim 2, [which presents]with more than 80%[, preferably more than 90%, more specifically more than 95%,] homology with the nucleotide sequence SEQ ID NO:1 or its complementary strand.
4. (Amended) [Isolated and purified genetic sequence]The isolated polynucleotide according to claim 1, [any one of the preceding claims, being the nucleotide sequence SEQ ID NO:1, its complementary strand or a portion thereof having more than 100 nucleotides and]wherein said polynucleotide [encoding]encodes a peptide [controlling]which positively and/or negatively controls the activation of a xylanase promoter-operator nucleotide sequence.
5. (Amended) [Isolated and purified genetic sequence]The isolated polynucleotide according to claim 1, [being]encoding an amino-acid sequence [which presents]having more than 60% homology with SEQ ID NO:2.
6. (Amended) [Isolated and purified genetic sequence]The isolated polynucleotide according to claim 5, [being]wherein the amino-acid sequence

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[which] presents more than 80%[, preferably more than 90%, more specifically more than 95%,] homology with SEQ ID NO:2.

7. (Amended) [Isolated and purified genetic sequence] The isolated polynucleotide according to claim 1, [being] encoding the amino-acid sequence SEQ ID NO 2 or a portion thereof having more than 50 amino-acids **[which is capable of controlling]** wherein said portion controls positively and/or negatively in trans the expression of a xylanase promoter-operator nucleotide sequence.
8. (Amended) [Nucleotide] A polynucleotide construct [(6)] comprising the isolated and purified polynucleotide [sequence] according to **[any one of the claims 1 to 4]** claim 1, operably linked to a xylanase promoter-operator polynucleotide [sequence (2) and possibly a nucleotide sequence (5) which is cis-activated by said xylanase promoter-operator nucleotide sequence (2)].
9. (Amended) [Vector (7), preferably a plasmid,] A vector comprising the isolated and purified polynucleotide [sequence (2)] according to **[any one of the claims 1 to 7 or the nucleotide construct (6) according to claim 8]** claim 1.
10. (Amended) [Cell] A cell transformed by the vector according to claim 9 **[and which allows the expression of the isolated and purified genetic sequence according to any one of the claims 1 to 7].**

Please add the following Claims:

11. The isolated polynucleotide according to claim 3, with more than 90% homology with the nucleotide sequence SEQ ID NO:1 or its complementary strand.
12. The isolated genetic sequence according to claim 3, with more than 95% homology with the nucleotide sequence SEQ ID NO:1 or its complementary strand.
13. The isolated polynucleotide of Claim 1 further comprising a cofactor.
14. The isolated polynucleotide of Claim 11 wherein said cofactor is selected from the group consisting of glucose, xylan, and mixtures thereof.
15. A method for the up-regulation or down-regulation of xylanase, comprising: providing the polynucleotide of Claim 1 to a cell which expresses xylanase.
16. The method of Claim 15, further comprising providing a cofactor.

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17. The method of Claim 16, wherein said cofactor is selected from the group consisting of glucose, xylan, and mixtures thereof.

18. The polynucleotide of Claim 1 wherein said xylanase promoter-operator is SEQ ID NO:2.

19. The polynucleotide of Claim 1 wherein said xylanase promoter-operator is from Streptomyces strain EC3 xInC.

20. A method for the production of antibiotics, malting of cereals, and production of paper, comprising

adding the polynucleotide of Claim 1 or the polypeptide encoded by the polynucleotide of Claim 1 to any cell which is producing any substance selected from the group consisting of: antibiotics, enzymes for malting of cereals, and enzymes for the production of paper.

21. The isolated polynucleotide according to claim 5, wherein the amino-acid sequence presents more than 90% homology with SEQ ID NO:2.

22. The isolated polynucleotide according to claim 5, wherein the amino-acid sequence presents more than 95%, homology with SEQ ID NO:2.

23. The polynucleotide of claim 8, further comprising a polynucleotide which is cis-activated by said xylanase promoter-operator nucleotide sequence.

24. The vector of claim 9 wherein said vector is a plasmid.

25. The isolated polynucleotide of Claim 1 comprising SEQ ID NO:3.

26. The isolated polynucleotide of Claim 5 comprising SEQ ID NO:4.

IN THE ABSTRACT

Please add the enclosed abstract page 16.

REMARKS

The Specification and Claims have been amended to conform to practice before the United States Patent and Trademark Office, to correct minor informalities such as European spellings of words. An abstract, corresponding to that in the PCT application has been added. Claims 11-27 have been added. Support for the added claims can be found in the original Claims as well as in the Specification. Specifically, support for added Claim 19 can be found on page 3, lines 33-34 and page 4, line 1. Support for added Claim 20 can be found on page 7, lines 29-34

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through page 8, line 1-3. As a result of the amendments made herein claims 1-27 are pending.
No new matter has been added herewith.

Conclusion

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 14 Feb. 2001

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ABSTRACT

The present invention is related to an isolated and purified genetic sequence controlling in trans the expression of a xylanase promoter-operator nucleotide sequence.

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NUCLEOTIDE AND/OR AMINO-ACID SEQUENCE CONTROLLING THE
EXPRESSION OF A XYLANASE PROMOTER-OPERATOR NUCLEOTIDE
SEQUENCE

Doc 1 >
Field of the invention

The present invention is related to a new
15 nucleotide sequence controlling in trans the expression of
a xylanase promoter-operator nucleotide sequence, the
amino-acid sequence encoded by said new nucleotide
sequence, the vector comprising said new nucleotide
sequence and the cell, preferably a *Streptomyces* strain,
20 transformed by said vector.

Background of the invention

In beer production, efficient hydrolysis of
xylans and other saccharides is important because said
25 compounds can be involved in production problems such as
wort viscosity (Ducroo, P. & Frelon, P.G., *Proceedings of*
the European Brewery Convention Congress, Zurich, 1989,
445; Viëtor, R.J. & Voragen, A.G.J., *Journal of the*
Institute of Brewing, 1993, 99, 243) and filterability and
30 haze formation (Coote N. & Kirsop, B.H., *Journal of the*
Institute of Brewing, 1976, 82, 34; Izawa, M., Kano, Y. &
Kanimura, M., *Proceedings Aviemore Conference on Malting,*
Brewing and Distilling, 1990, 427).

In other areas, efficient hydrolysis of xylans and/or arabinoxylans is highly desirable as well. Examples include rye and wheat breadmaking processes, paper and pulp technologies (see US patent 5,116,746). It follows
5 that a lot of research efforts have been devoted to the xylan hydrolysis enzymes due to their applications as described above.

Aims of the present invention

10 The aim of the present invention is to provide a method and system which improve the control upon the expression of nucleotide sequence encoding enzymes such as xylanase, as well as homologous or heterologous sequences of said enzymes whose transcription is also
15 activated by a xylanase promoter-operator regulatory sequence.

A specific aim of the present invention is to provide such a method and system for improving enzymatic processes, especially for improving production of
20 antibiotics, malting processes of cereals such as barley, sorghum and wheat, production of beers, of baked or extruded cereals products, animal feed stuff, the production of starch derived from syrups, sorbitol, xylose and xylitol, and for the improvement of paper and pulp
25 technologies.

Summary of the invention

The present invention is related to a new nucleotide sequence 1 which controls the expression of any
30 xylanase promoter-operator nucleotide sequence 2. Said control upon the activation of a xylanase promoter-operator nucleotide sequence 2 is advantageously obtained by trans-activation (said new nucleotide sequence 1 encoding a

trans-activated factor which controls the activation of said xylanase promoter-operator nucleotide sequence 2).

Therefore, the present invention is also related to said factor, preferably a peptidic factor 3 which is an activator and/or repressor encoded by said nucleotide sequence 1 and which controls positively and/or negatively the expression of a xylanase promoter-operator nucleotide sequence 2.

Advantageously, said factor could be present in a composition with other cofactors 4 that induce positively and/or negatively said mechanism.

Preferably, said cofactors 4 present in said composition are selected from the group consisting of glucose, xylan or a mixture thereof.

The Inventors have discovered unexpectedly that the presence of glucose induces a repressive mechanism upon the activation of a xylanase promoter-operator nucleotide sequence, while the presence of xylan induces a positive mechanism of said expression. The simultaneous presence of said two cofactors in a medium induces also positively the expression of a xylanase promoter-operator nucleotide sequence.

It is meant by "a xylanase promoter-operator nucleotide sequence", any nucleotide sequence 2 which activates any nucleotide sequence 5 encoding a xylanase enzyme.

A classification of the xylanase enzymes in the categories F/10 and G/11 is described by Henrissart et al. (Biochem. J. 293, pp. 781-788).

Said xylanase promoter-operator nucleotide sequences comprise at least one 5 base pair pattern : 5'-CGAAA-3'.

Preferably, said xylanase promoter-operator nucleotide sequence is the *Streptomyces* sp. strain EC3 xlnC

xylanase promoter-operator nucleotide sequence SEQ ID NO 2 also described by Giannotta F. et al. (*FEMS Microbiol. Letters* 142, pp. 91-97 (1996)).

According to a preferred embodiment of the present invention, the isolated and purified nucleotide sequence according to the invention is a (DNA) sequence which presents more than 60%, advantageously more than 80%, preferably more than 90%, and more preferably more than 95%, homology (i.e. sequence identity) with the nucleotide sequence SEQ ID NO 1 or its complementary strand described hereafter.

According to another preferred embodiment of the present invention, said isolated and purified nucleotide sequence corresponds to the nucleotide sequence SEQ ID NO 1 or its complementary strand or a portion thereof; preferably a sequence having more than 100 nucleotides and encoding a peptide which still controls positively and/or negatively the expression of a xylanase promoter-operator nucleotide sequence.

Preferably, said sequence portion comprises at least the nucleotides of SEQ ID NO 3 or any nucleotide sequence encoding for its corresponding peptidic sequence.

According to a further preferred embodiment of the present invention, the terms "a portion of the nucleotide sequence SEQ ID NO 1 or its complementary strand" mean any kind of nucleic acid molecule (DNA, RNA, antisense nucleotide sequence, etc.) which is specific of SEQ ID NO 1, comprises more than 15 nucleotides (such as a probe or one or several primers), and which may be used to identify, reconstitute or block the transcription of said specific isolated and purified nucleotide sequence SEQ ID NO 1 or its complementary strand. Said identification, reconstitution or blocking is obtained with known techniques by the person skilled in the art, such as the

use of antisense RNA, specific labelled probe hybridisation or genetic amplification, preferably by PCR (as described in the US patent 4,965,188) or by LCR (as described by Landgren et al. (*Sciences* **241**, pp. 1077-1080 (1988))).

5 Therefore, the present invention is also related to any nucleotide sequence which presents an homology (i.e. sequence identity) as above-described with SEQ ID NO 1, SEQ ID NO 3 or their complementary strands, or any nucleotide sequence which preferably allows an
0 ~~hybridisation~~ hybridisation with SEQ ID NO 1, SEQ ID NO 3 or their complementary strands under standard stringent hybridisation conditions, and which may encode the same or a similar amino-acid sequence due to the redundancy of the genetic code.

15 Exemplary standard stringent hybridisation
conditions are as follows : hybridisation at 40 °C in 50%
formamide, 5x SSC, 20 mMol sodium phosphate, pH 6.8,
washing in 0.2x SSC at 50 °C. Variations in these
conditions may occur based on the length and the GC
20 nucleotide content of the sequence to be hybridised.
Formula standard in the art are approved for determining
exact hybridisation conditions such as the one described by
Sambrook et al. (Molecular Cloning : A Laboratory Manual,
Cold Spring Harbor, Laboratory Press, Cold Spring Harbor,
25 New York, \$9.47-9.51 (1989)).

Another aspect of the present invention is related to the amino-acid sequence encoded by said nucleotide sequence, and which present more than 60%, advantageously more than 80%, preferably more than 90%, more preferably more than 95% homology (i.e. sequence identity), with SEQ ID NO 2.

According to another embodiment of the present invention, the amino-acid sequence according to the

invention corresponds to the amino-acid sequence of SEQ ID NO 2 or any portion thereof having preferably more than 50 amino-acids and which is still capable of controlling (positively or negatively) in trans the expression of a xylanase promoter-operator nucleotide sequence.

Preferably, said portion is an amino-acid sequence that comprises at least the amino-acid sequence encoded by the nucleotide sequence SEQ ID NO 3 above-described.

It is meant by "controlling (positively and/or negatively) in trans the expression of a xylanase promoter-operator nucleotide sequence", the possibility for any nucleotide sequence 1 or any amino-acid sequence 3 encoded by said nucleotide sequence 1 to induce or reduce (preferably in the presence of the other cofactors 4 such as glucose and/or xylan) the expression of a xylanase promoter-operator nucleotide sequence 2 and obtain thereafter a control upon the cis-activation of a downstream nucleotide sequence 5 (for instance a gene encoding a xylanase enzyme) which is controlled in cis by said xylanase promoter-operator nucleotide sequence. The inducing or reduction of said expression is observed preferably by a positive or a negative modification of said cis-activation (for instance by an increasing or a decreasing of the synthesis of said xylanase enzyme by a cell). Said mechanism is also illustrated in the enclosed Fig. 3.

The present invention is also related to a nucleotide construct 6 comprising the isolated and purified nucleotide sequence 1 according to the invention, linked to a xylanase promoter-operator nucleotide sequence 2 and possibly any homologous or heterologous nucleotide sequence 5 of a gene encoding a xylanase enzyme, which is cis-

activated by said xylanase promoter-operator nucleotide sequence 2.

Another aspect of the present invention is related to the vector 7 comprising said isolated and
5 purified nucleotide sequence 1 or the nucleotide construct 6 according to the invention. Advantageously, said vector 7 is a plasmid comprising the necessary elements (origin of replication ORI) for the transfection of said nucleotide sequence 1 or said nucleotide construct 6 into a cell,
10 preferably into a *Streptomyces* sp. strain.

The vector according to the invention may comprise also other elements, such as a marker (thiostreptone = tsr) for the identification of a possible transformation by the vector according to the invention in
15 said specific cell. The vector according to the invention can be also a shuttle vector comprising the necessary elements for the expression of said shuttle vector in *E. coli* and *Streptomyces* sp.

Another aspect of the present invention is
20 related to the cell such as a gram-positive bacteria, preferably a *Streptomyces* strain, transformed by said vector 7 or by said shuttle vector, which allows the expression of the isolated and purified nucleotide sequence 1 according to the invention controlling the activation of
25 the xylanase promoter-operator nucleotide sequence 2 present in said cell and therefore the transcription of any nucleotide sequence 5 which could be cis-activated by said xylanase promoter-operator nucleotide sequence 2.

The nucleotide construct 6, the vector 7
30 and/or the cell transformed by said vector as well as specific portions of the isolated and purified nucleotide sequence 1 according to the invention can be advantageously used in several industrial biochemical processes such as production of antibiotics, malting processes of cereals,

preparation of beers, baked or extruded cereals products, for the improving of animal feed stuff and for the improvement of paper and pulp technologies.

The products of the invention, possibly combined with the above-described cofactors, are advantageously present in a bioreactor, and will allow the controlled synthesis of proteins or peptides of interest or possibly avoid or reduce the synthesis of said proteins or peptides by specific cells in the above-identified biochemical industrial processes.

The various aspects of the present invention will be described in details in the enclosed non-limiting examples in reference to the following figures.

15 Brief description of the drawings

Figures 1 to 3 represent the steps for the construction of the vector according to the invention.

Detailed description of the invention

20 The alignment of various nucleotide sequences upstream xylanase gene in the strain *Streptomyces* sp. EC3, shows the presence of three repetitive units of five BP : 5'-CGAAA-3' observed among all xylanase sequences (except in the strain *Actinomadura* sp. which comprises only one repetitive unit).

In the specific strain *Streptomyces* sp. EC3, three boxes in the promoter-operator regions of 390 BP are defined : box 1 (B1) at -200 BP, box 2 (B2) at -210 BP and box 3 (B3) at -350 BP from the ATG codon. The box B3 is extremely conserved between the *Streptomyces* strain. (83% of identity of sequence upon 12 bases).

The identification of the repetitive consensus sequence is presented in the following table 1.

Table 1

Cons. B1	C G A A A C T G T T G A	[SEARCH NO: 7]
Cons. B2	T T T C C G A A A G T T T G C	[SEARCH NO: 8]
Cons. B3	T C G A A A C T T T C G	[SEARCH NO: 9]
5 Consensus	t C G A A A g c	[SEARCH NO: 10]

However, it seems that said consensus
 nucleotide sequence ~~TXXCGAAAXXGXGXC~~ [SEARCH NO: 10]
 is not present in other known xylanase
 nucleotide sequence of other bacteria such as *Bacillus*
 10 strains.

The Inventors have discovered that the
 proteinic trans-activation factor according to the
 invention affects the regulation of said specific portions
 (B3 > B2 > B1) of the xylanase promoter-operator nucleotide
 15 sequence of the *Streptomyces sp.* EC3.

The Inventors have also discovered a
 modification of the trans factor affinity for the B2 box in
 repression and induction.

Repression : B3 > B2 > B1

20 Induction : B3 >> B2 = B1

Additional competitive experiments have
 identified as a preferred fixation site of the trans-
 activation factor according to the invention, the above-
 identified specific regions (boxes 3, 2 and 1).

25 It should be noted also that the above-
 described boxes present inverse repeated sequence and a
 palindrome of 4 BP that seems to be specifically recognised
 by the proteinic trans-activation factor according to the
 invention.

30 Therefore, it seems that the main fixation
 site of said proteinic trans-activation factor is the box
 B3, which allows thereafter a fixation upon the box B2 even
 when a mutation is present in said box B2.

According to said preliminary results, it seems that the control upon the activation of a xylanase gene is based upon operative sites which are specifically recognised by a trans-activation factor which is working as a repressor and which allows the formation of a repressive loop (connection between the B2 and B3 boxes by the trans-activation factor) and avoids the fixation of the RNA-polymerase and thereafter the transcription of a downstream coding nucleotide sequence.

10 Genetic identification of the proteinic trans-activation factor and its encoding nucleotide sequence

The gene coding for xylanase C of *Streptomyces* sp. has been cloned into a multicopy vector which confers positive xylanase phenotype when the host strain is under repression conditions. Repressed clones, which may be a genomic fragment encoding the repressor according to the invention, will be characterised by a wild type phenotype.

Repressors from a genomic bank in the vector pDML614 were isolated.

After plasmid purification, an amplification by PCR allows a raw estimation of the insert size, which is presented in Table 2.

25	<u>PCR conditions</u> :	Step 1 :	96 °C	4 min
		Step 2 :	30 cycles	94 °C 30 sec
		Step 3 :	54 °C	1 min
		Step 4 :	72 °C	3 min 30 sec
		Step 5 :	72 °C	10 min
30		Step 6 :	4 °C	

Table 2 : Size of the insert

Clone	Size of the PCR product (kb)	Estimated size of the insert (kb)
S1	2,5	0,9
S2	3,2	2,5
S3	2,5	0,9
S4	1,6	0,1
S5	2,1	0,5
S6	2,8	1,2
pDML614	1,6	0

A sequence of 1022 nucleotides obtained from the clone S6 allows the identification of an open reading frame with several bacterial regulator systems. A first polypeptide of 164 amino acids was identified and the corresponding nucleotide sequence was used as a probe for the isolation of the complete nucleotide sequence SEQ ID NO 1.

The cloning of the carboxy terminal portion was obtained by Southern blotting. 2,5 genomic DNA of *Streptomyces* sp. EC3 are cleaved by several restriction enzymes and have been transferred upon a nylon membrane. A fragment of 720 BP has been amplified and labelled with biotiny by PCR, and is used as a probe for the specific hybridisation of the genomic DNA. A portion of the genomic DNA of *Streptomyces* sp. was cleaved by restriction enzymes and the generated fragments by PCR were introduced in a plasmid pUC for sequencing.

The sequenced nucleotide sequence comprises four open reading frames. The longest open reading frame hereafter called xlnR was implicated in the regulation of the xylanase enzyme, and the corresponding amino-acid sequence was identified by the BLAST software.

The complete isolated and purified nucleotide sequence 1 according to the invention was introduced in a vector 7 having incorporated also a xylanase promoter-operator nucleotide sequence 2 linked to a gene encoding a xylanase enzyme 5. Advantageously, said xylanase promoter-operator nucleotide sequence 2 comprises a poly-linker sequence (nucleotide sequence with several cleaving sites) which improves the insertion of homologous or heterologous sequences. The characteristics of the vector according to the invention were improved by incorporating a specific marker (such as the thiostreptone) which is used for the specific selection of transformed cells.

The vector according to the invention was advantageously a shuttle vector comprising the necessary elements for the transfection of said vector in a *Streptomyces* strain and in *E. coli* (see also U.S. patent 4,992,371 incorporated hereafter by reference).

Preferably, said shuttle vector was prepared according to the method comprising the following steps. The pUC18 polylinker was replaced by a dsDNA fragment containing endonuclease restriction sites and the following dsDNA fragment was entered in a *Hind*III-*Eco*RI-digested pUC18 (L08752, Norrander et al., Gene 26, pp. 101-106 (1983)).

dsDNA fragment: SEQ ID NO: 6: 5'- AGC TAG GCC TAT CGA TGG CGC GCC AAG CTA GCA ACT TAA GTA GAT CTA ACT AGT CTG CAG CAG AAG CTT AAT ATT TAA TTA AGC GGC CGC AGT ACT CTC GAG CCG CCA TGG GCC CGA TAT CGG TAC CAG GCC T- 3' (or SEQ ID NO: 5)
(Endonuclease restriction sites: 5'-*Cla*I-*Asc*I-*Nhe*I-*Afl*III-*Bgl*II-*Spe*I-*Pst*I-*Hind*III-*Ssp*I-*Pac*I-*Not*I-*Sca*I-*Xho*I-*Nco*I-*Apa*I-*Eco*RV-*Kpn*I-3').

Thereafter, the streptomycin/spectinomycin resistance gene (Str/Spm) from an omega interposon

(Prentki, P. & Krisch, H.M. Gene 29 pp. 303-313 (1984)) was introduced at the *HindIII* restriction site.

The pUC18 sequence was deleted from the construction and replaced by the *ClaI-KpnI Streptomyces* 5 replication origin from the pIJ702 vector (Katz et al., J. Gen. Microbio. 129 pp. 2703-2714).

The construction was achieved to be a shuttle vector: a 1242 bp *AseI-NdeI* DNA fragment, containing the *E. coli* DNA replication origin from the pBR322 vector (J01749, 10 Sutcliffe, J.G., Proc. Natl. Acad. Sci. U.S.A. 75(8), pp. 3737-3741 (1978)) was treated by klenow and introduced in *EcoRV*.

The regulatory sequence *xlnR* was introduced in a *PacI-ScaI*-digested vector and the *xlnC* structural gene 15 with its promoter in the *AscI-PstI* restriction sites in order to obtain the shuttle vector "Vpro" according to the invention (see enclosed Fig. 3).

For the analysis of an heterologous expression of foreign~~er~~ genes in *Streptomyces*, the person 20 skilled in the art may refer to the US patent 5,641,663 and the US patent 5,435,730.

Furthermore, the vector according to the invention may also comprise one or more mutations in the xylanase promoter-operator nucleotide sequence 2 in order 25 to improve (increase) a cis-activating by said xylanase promoter-operator nucleotide sequence.

H 26 07 00

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WHAT IN THE CLAIMS
CLAIMS

Seal → 1. Isolated and purified bacterial genetic sequence (1) controlling in trans the expression of a xylanase promoter-operator bacterial nucleotide sequence (2).

2. Isolated and purified genetic sequence according to claim 1, being a nucleotide sequence which presents more than 60% homology with the nucleotide sequence SEQ ID NO 1 or its complementary strand.

3. Isolated and purified genetic sequence according to claim 2, which presents more than 80%, preferably more than 90%, more specifically more than 95%, homology with the nucleotide sequence SEQ ID NO 1 or its complementary strand.

4. Isolated and purified genetic sequence according to any one of the preceding claims, being the nucleotide sequence SEQ ID NO 1, its complementary strain or a portion thereof having more than 100 nucleotides and encoding a peptide controlling positively and/or negatively the activation of a xylanase promoter-operator nucleotide sequence.

5. Isolated and purified genetic sequence according to claim 1, being an amino-acid sequence which presents more than 60% homology with SEQ ID NO 2.

6. Isolated and purified genetic sequence according to claim 5, being an amino-acid sequence which presents more than 80%, preferably more than 90%, more specifically more than 95%, homology with SEQ ID NO 2.

M 28 07 .00

2

7. Isolated and purified genetic sequence according to claim 1, being the amino-acid sequence SEQ ID NO 2 or a portion thereof having more than 50 amino-acids which is capable of controlling positively and/or
5 negatively in trans the expression of a xylanase promoter-operator nucleotide sequence.

8. Nucleotide construct (6) comprising the isolated and purified nucleotide sequence according to any one of the claims 1 to 4, linked to a xylanase promoter-
10 operator nucleotide sequence (2) and possibly a nucleotide sequence (5) which is cis-activated by said xylanase promoter-operator nucleotide sequence (2).

9. Vector (7), preferably a plasmid, comprising the isolated and purified nucleotide sequence
15 (2) according to any one of the claims 1 to 7 or the nucleotide construct (6) according to claim 8.

10. Bacterial cell transformed by the vector according to claim 9 and which allows the expression of the isolated and purified genetic sequence according to any one
20 of the claims 1 to 7.

add
A2

PCT

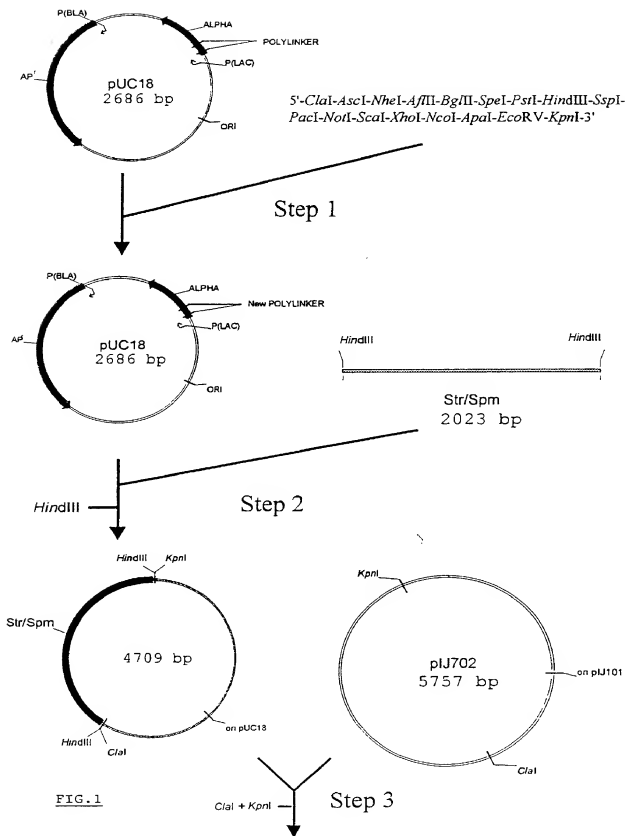
WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification 7 : C12N 15/76, 9/24, 1/21, C07K 14/36</p>	<p>A1</p>	<p>(11) International Publication Number: WO 00/09717 (43) International Publication Date: 24 February 2000 (24.02.00)</p>
<p>(21) International Application Number: PCT/BE99/00105 (22) International Filing Date: 12 August 1999 (12.08.99) (30) Priority Data: 60/096,556 14 August 1998 (14.08.98) US (71) Applicant (for all designated States except US): UNIVERSITE DE LIEGE [BE/BE]; Place du XX Août 7, B-4000 Liège (BE). (72) Inventors; and (75) Inventors/Applicants (for US only): GIANNOTTA, Fabrizio [BE/BE]; Rue de la Liberté 38, B-4020 Liège (BE). RIGALI, Sébastien [BE/BE]; Rue Président Kennedy 75, B-4420 Montegnée (BE). DUSART, Jean [BE/BE]; Rue d'Esneux 19, B-4450 Nandrin (BE). (74) Agents: VAN MALDEREN, Eric et al.; Office Van Malderen, Place Reine Fabiola 6/1, B-1083 Brussels (BE).</p>		<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report.</p>
<p>(54) Title: NUCLEOTIDE AND/OR AMINO-ACID SEQUENCE CONTROLLING THE EXPRESSION OF A XYLANASE PROMOTER-OPERATOR NUCLEOTIDE SEQUENCE</p> <p>(57) Abstract</p> <p>The present invention is related to an isolated and purified genetic sequence (1) controlling in trans the expression of a xylanase promoter-operator nucleotide sequence (2).</p>		

SCANNED, # 12



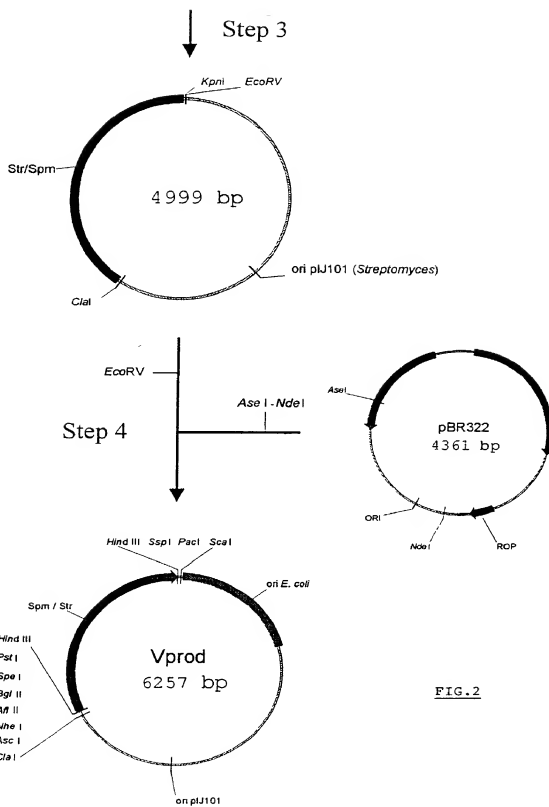


FIG. 2

Final Step

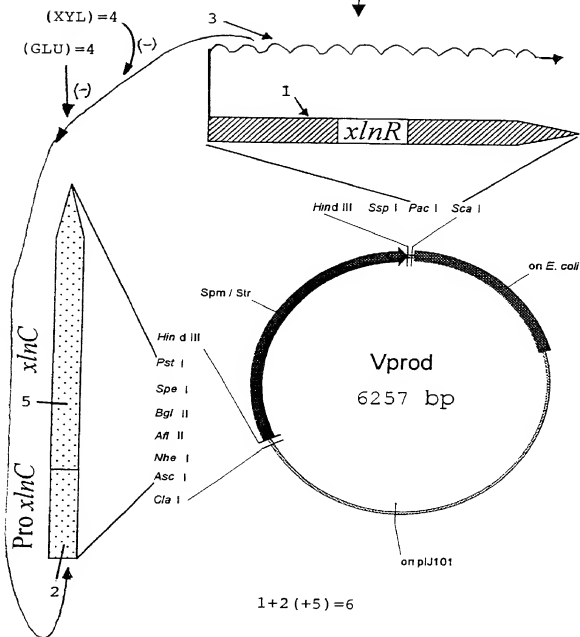


FIG. 3

Declaration and Power of Attorney for Patent Application**Déclaration et Pouvoirs pour demandes de brevet****French Language Declaration**

En tant que l'inventeur nommé ci-après, je déclare par le présent acte que :

Mon domicile, mon adresse postale et ma nationalité figurant ci-dessous à côté de mon nom,

Je crois être le premier inventeur original et unique (si un seul nom est mentionné ci-dessous), ou l'un des premiers co-inventeurs originaux (si plusieurs noms sont mentionnés ci-dessous) du sujet revendiqué, pour lequel une demande de brevet a été déposée concernant l'invention intitulée :

et dont les caractéristiques sont fournies ci-joint à moins que la case suivante n'ait été cochée :

- ☐ a été déposé le
sous le numéro de Demande des Etats-Unis ou
sous le numéro de demande internationale
PCT et modifiée le
(le cas échéant).

Je déclare par le présent acte avoir passé en revue et pris connaissance du contenu des caractéristiques ci-dessus, revendications comprises, telles que modifiées par tout amendement dont il aura été fait référence ci-dessus.

Je reconnais de voir divulguer toute information pertinente à l'examen de cette demande, comme le définit le Titre 37, §1.56 du Code fédéral des réglementations.

As a below named inventor, I hereby declare that :

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled :

**NUCLEOTIDE AND/OR AMINO-ACID
SEQUENCE CONTROLLING THE
EXPRESSION OF A XYLANASE
PROMOTER-OPERATOR NUCLEOTIDE
SEQUENCE.**

the specification of which is attached hereto unless the following box is checked :

- ☒ was filed on **Herewith**
as United States Application Number or PCT
International Application Number
PCT/BE99/00105 on 12.08.99 (filed 8/14/99)
and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

French Language Declaration

Je revendique par le présent acte avoir la priorité étrangère, en vertu du Titre 35, § 119 du Code des Etats-Unis, sur toute demande étrangère de brevet ou certificat d'inventeur figurant ci-dessous et ai aussi pris connaissance de toute demande étrangère de brevet ou de tout certificat d'inventeur ayant une date de dépôt précédant celle de la demande à propos de laquelle une priorité est revendiquée.

Prior foreign applications

Demande(s) de brevet antérieure(s)

(Number)	(Country)
(Numéro)	(Pays)
60/096,556	US
(Number)	(Country)
(Numéro)	(Pays)
(Number)	(Country)
(Numéro)	(Pays)

Je revendique par le présent acte tout bénéfice, en vertu du Titre 35, § 120 du Code des Etats-Unis, de toute demande de brevet effectuée aux Etats-Unis figurant ci-dessous et, dans la mesure où le sujet de chacune des revendications de cette demande de brevet n'est pas divulgué dans la demande américaine préalable, en vertu des dispositions de premier paragraphe du Titre 35, § 112 du Code des Etats-Unis, je reconnais devoir divulguer toute information pertinente à la demande de brevet comme défini dans le Titre 37, § 1.56 du Code fédéral des réglementations, dont j'ai pu disposer entre la date de dépôt de la première demande et la date de dépôt de la demande nationale ou PCT internationale :

(Application Serial No.)	(Filing date)
(No. de série de la demande)	(Date de dépôt)

(Application Serial No.)	(Filing date)
(No. de série de la demande)	(Date de dépôt)

Je déclare par le présent acte que toute déclaration ci-incluse est, à ma connaissance, véridique et que toute déclaration formulée à partir de renseignements ou de suppositions est tenue pour véridique; et de plus, que toutes ces déclarations ont été formulées en sachant que toute fausse déclaration volontaire ou son équivalent est passible d'une amende ou d'une incarcération, ou des deux, en vertu de la Section 1001 du Titre 18 du Code des Etats-Unis et que de telles déclarations volontairement fausses risquent de compromettre la validité de la demande de brevet ou du brevet délivré à partir de celle-ci.

I hereby claim foreign priority under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Priority claimed
Droit de priorité revendiqué

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(Day/Month/Year Filed)	Yes	No
(Jour/Mois/Année de dépôt)	Oui	Non
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(Day/Month/Year Filed)	Yes	No
(Jour/Mois/Année de dépôt)	Oui	Non

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application :

(Statut)	(Status)
(Breveté, en attente, annulé)	(Patented, pending, abandoned)

(Statut)	(Status)
(Breveté, en attente, annulé)	(Patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful and false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

French Language Declaration

POUVOIRS : En tant que l'inventeur cité, je désigne par la présente l'(les) avocat(s) et/ou agent(s) suivant(s) pour qu'il(s) poursuive(nt) la procédure de cette demande de brevet et traite(nt) toute affaire avec le Bureau des brevets et marques s'y rapportant.
(mentionner le nom et le numéro d'enregistrement)

POWER OF ATTORNEY : As named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and trademark Office connected there with.

(list name and registration number)

Adresser toute correspondance à :

Send Correspondence to :

Adresser tout appel téléphonique à :
(nom et numéro de téléphone)

Direct Telephone Calls to :
(name and telephone number)

Nom complet de l'unique ou premier inventeur	Full name of sole or first inventor	
Signature de l'inventeur	Date	Inventor's signature Date
Domicile	Residence	
Nationalité	Citizenship	
Adresse postale	Post Office Address	

GIANNOTTA Fabrizio
8th January 2001
BE
Rue de la Liberté 38
B-4020 LIEGE
BELGIUM

(Fournir les mêmes renseignements et la signature de tout co-inventeur supplémentaire)

(Supply similar information and signature for any subsequent joint inventor)

2^e

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Signature du second inventeur	RIGALI Sébastien Second inventor's signature
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Domicile	Residence Rue Président Kennedy 75 B-4420 MONTEGNEE BELGIUM B E L
Nationalité	Citizenship Belgian
Adresse postale	Post Office Address Rue Président Kennedy 75 B-4420 MONTEGNEE BELGIUM

3^e

Nom complet du troisième co-inventeur, le cas échéant	Full name of third joint inventor, if any
Signature du second inventeur	DUSART Jean Third inventor's signature
Date	Date 8/1/01
Domicile	Residence Rue d'Esneux 19 B- 4450 NANDRIN BELGIUM B E L
Nationalité	Citizenship Belgian
Adresse postale	Post Office Address Rue d'Esneux 19 B- 4450 NANDRIN BELGIUM

Nom complet du quatrième co-inventeur, le cas échéant	Full name of fourth joint inventor, if any
Signature du second inventeur	Third inventor's signature
Date	Date
Domicile	Residence
Nationalité	Citizenship
Adresse postale	Post Office Address

SEQUENCE LISTING

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